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Basic Investigation

Inhibition of hypoxia and serum deprivation-induced apoptosis by salvianolic acid in rat mesenchymal stem cells

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Abstract

OBJECTIVE: To investigate the influence and mechanism of salvianolic acid B (SalB) on apoptosis inhibition in rat bone marrow-derived mesenchymal stem cells (BMSCs) induced by hypoxia and serum deprivation (hypoxia/SD).

METHODS: SalB concentration of 0.1, 1, 10 or 100 mg/L (drug groups) were investigated for their ability to inhibit apoptosis in rat BMSCs. BMSCs in both the apoptosis model and drug groups were cultured under hypoxic conditions for 6 h, after which cell apoptosis and change in mitochondrial membrane potential (MMP) were detected using flow cytometry. Activation of caspase-3 was detected using western blot analysis.

RESULTS: Hypoxia/SD induced apoptosis in rat BMSCs. The early apoptosis rate was lower in the drug groups compared to the apoptosis model group ($P<0.05$). SalB was found to inhibit the reduction in MMP and decrease the activation of caspase-3.

CONCLUSION: 0.1, 1 and 10 mg/L of SalB inhibits activation of caspase-3 and early apoptosis of rat BMSCs induced by hypoxia/SD and could therefore enhance the survival rate of grafted stem cells.

Key words: SalB; BMSCs; Hypoxia/SD; Apoptosis

INTRODUCTION

Myocardial cell apoptosis and cardiac function failure, caused by ischemic heart disease, are common causes for mortality. Bone marrow-derived mesenchymal stem cell (BMSC) grafting has good prospects for clinical application in repairing cardiac muscle and restoring cardiac function. However, low survival rate of grafted BMSCs [1,2] greatly restricts the use of this therapeutic approach. Danshen (Radix Salviae Miltiorrhizae) has good curative effects on various types of ischemic heart disease. SalB, a main ingredient of Danshen, was used to investigate the mechanism of Danshen in the body. In this research, hypoxia/SD was induced to mimic the micro-environment of ischemic cardiac muscle in order to promote apoptosis of rat BMSCs, in order to determine the effect and mechanism of SalB apoptosis inhibition in BMSCs.

MATERIALS AND METHODS

Reagents and instruments

SalB (Nanjing Tisiaime), Iscove's Modified Dulbecco's

Medium (IMDM, Gibco BRL), excellent grade ox fetus serum (Gibco BRL), trypsase (Gibco, USA), Hoechst 33342 (Sigma), reagent kit for detecting apoptosis of Annexin V-FITC cells (Baosai Biological, Beijing), hypoxia-culturing kit (Bio Mérieux Rsa, France), Rhodamine 123 (Sigma), reagent kit for detecting activation of caspase-3 enzyme (Biovision), caspase-3 antibody, cleaved caspase-3 antibody and actin antibody (Santa Cruz, USA), horse radish peroxidase (Zhongshan Biological Limited, Beijing), cell-culturing box for CO_2 at constant temperature (Jouan, France), fluorescence/inverted microscope (Olympus, Japan) and flow cytometry (BD Biosciences, USA).

Isolation and culture of BMSCs

The femur and tibia from a Sprague Dawley (SD) rat were excised and cells were slowly flushed out from the marrow cavity using culture medium prior to seeding into a 75 cm^2 culture flask. After 24 h, culture medium was replaced. After 4-5 days culture and on reaching 70%-80% confluence, original passage BMSCs were passaged at a ratio of 1:3 to generate second passage cells. Cultured rat BMSCs were observed under an inverted microscope.

Determining the growth curve

The BMSCs (the second passage, P2) were seeded into 24 well plates at a density of 5000 cells/well. Cell counts were carried out at days 2, 3, 4, 5, 6, 7 and 8 after seeding. Cells from 3 wells were used to determine each cell count and mean values were used to determine the growth curve.

Experimental groups

Same passage BMSCs with similar growth characteristics were obtained from 6 flasks and were randomly divided into the following 6 groups: control group (complete IMDM containing 15% fetal bovine serum), apoptosis model group (serum-free IMDM) and 4 SalB treatment groups (serum-free IMDM with either 0.1, 1, 10 or 100 mg/L SalB). In the SalB treatment groups, cells were cultured for 1 h with culture complete medium containing the relevant concentration of SalB. After an hour, cells were rinsed twice with phosphate buffered saline (PBS) and exposed to experimental condition for different concentrations SalB (0.1, 1, 10 or 100 mg/L) for 6 h in hypoxia/SD conditions. The apoptosis model group and SalB treated groups were put into boxes for culturing cells under hypoxic conditions. After addition of a hypoxia catalyst, boxes were immediately closed. Both the control group and hypoxia-culture boxes were incubated in 5% CO_2 at 37°C for 6 h.

Hoechst staining for observation of cell apoptosis using inverted and fluorescence microscopy

Cells in the 6 groups were rinsed twice with PBS. After addition of culture medium, Hoechst 33342 was added at a concentration of 0.1 mg/mL. Cells were protected from light and left at room temperature (r.t) for 10 min. An inverted microscope was used for observation and photographs were obtained. A fluorescence microscope was used to observe the state of cell apoptosis and photographs were obtained.

Annexin V/ Propidium iodide (PI) staining to detect cell apoptosis using flow cytometry

Cells in the 6 groups were centrifuged at the speed of 1000 rpm for 5 min at 4°C. Supernatants were discarded and cells were suspended in PBS. This procedure was repeated twice. Cells were suspended in 200 μL Binding Buffer. 10 μL Annexin V-FIFC was added in dark conditions at r.t for 15 min. 300 μL of Binding Buffer and 5 μL of PI was added. Within 10 min, flow cytometry was used for detection of living cells, early-, moderate- and late-apoptotic cells and necrotic cells.

Rhodamine 123 dye to determine MMP ($\Delta\psi m$)

SalB-treated cells were collected, rinsed twice with PBS and exposed to rhodamine 123 (10 mg/L) at 37°C for 30 min. MMP level was detected using flow cytometry.

Western blot to detect protein-expression level

SalB-treated cells were collected and protein was extracted. The Coomassie brilliant blue method was used to detect the concentration of protein. SDS polyacrylamide gel electrophoresis was used to separate proteins (50 g of sample per hole). Protein was electrically transferred to a fibrin nitrate membrane. Western blot analysis was used to detect the level of expression of the activated section of caspase-3. The fibrin nitrate membrane was immersed in TBST containing 5% skimmed milk powder at 4°C overnight, rinsed with TBST and exposed for one hour at r.t to the corresponding primary antibody. The membrane was rinsed 3 times for 10 min each at r.t with slow agitation in TBST. The membrane was exposed for 90 min at r.t to secondary antibody conjugated to horse radish peroxidase. After rinsing, the membrane was developed in chemical luminescence liquid, exposed onto X-ray film in a dark room and fixed to observe target protein.

Statistical analysis

All the data are expressed as mean ± standard deviation (SD). A non-paired t test was used. $P<0.05$ was regarded as a statistically significant difference.

RESULT

BMSC morphology

As shown in Figure 1, 24 h after cell seeding, a small number of cells were observed growing on the flask surface with extensions stretched out. Other cells with a round, bright appearance, in absence of adhesion to the surface, were also observed. With increased culture

time, cells gradually stretched to become more elongated and the colony quantity increased. On day 3 of culture, the density of elongated cells and cell colony number was further increased. By day 5 of culture, the cell volume was expanded, colonies were linked and confluence was about 70%-80%. Whirlpool-shaped growth was observed, typical of BMSCs. Cell growth morphologies of the original, second, fourth and fifth passages on day 5 were observed and compared. Second passage cells grew evenly on the surface with elongated morphology. Third and fourth passage cells were similar in appearance, although blunter. The morphology of fifth passage cells were not as elongate as other passage cells's. Subsequent experiments therefore used second, third and fourth passage BMSCs.

Growth curve of BMSCs

Figure 2 shows the growth curve of second passage BMSCs. On day 1, cells were in the latent period; on days 2-5, cell proliferation accelerated; on days 6 and 7, cell growth gradually decelerated and cell growth began to decline. All cells in the testing were on day 3-4 when the cell density reached 80%.

Exposure of SalB to BMSCs enables apoptosis inhibition after hypoxia/SD induction

Observation of SalB apoptosis inhibition of BMSCs under inverted microscope: Figure 3 shows that control group cells grew vigorously with an elongated morphology, in a regular form and with a whirlpool appearance. Cells in the apoptosis model group (hypoxia/SD) and 100 mg/L SalB-treated group, were irregular, wrinkled, unsaturated and had shrunk. The number of cells adhering to the surface was obviously decreased and there was a large number of floating dead cells. Cells in the groups treated with 0.1, 1 and 10 mg/L SalB, were regular and elongated in morphology, with the typical whirlpool appearance, in absence of large numbers of floating dead cells. These results indicate that compared to the apoptosis model group, BMSCs treated with 0.1, 1 and 10 mg/L SalB are able to resist apoptosis induced by hypoxia/SD. However, the BMSCs treated with 100mg/L SalB were unable to resist apoptosis.

Observation of BMSCs apoptosis resistance with SalB using Hoechst 33342 dye and fluorescence microscopy: As shown in Figure 4, the karyon in cells from the control group is large with uniform quality and light blue color. In cells from the apoptosis model and 100 mg/L SalB-treated group, the karyon was shrunk and fragmented with irregular form and dark color. In cells from the groups treated with 0.1, 1 and 10 mg/L SalB, the karyon is similar to that in the control group and the number of dead karyons is obviously decreased.

Annexin V/PI double dyed flow cytometry used to detect apoptosis resistance in BMSCs by SalB: As shown in Figure 5, after 6 h treatment with hypoxia/SD in the apoptosis model group, the number of surviving cells obviously decreased and early apoptosis increased (32.01 ± 4.16, P=0.00027). After treatment with SalB, the number of early apoptotic cells was reduced. In the groups treated with 0.1, 1, 10 and 100 mg/L SalB, the early cell apoptosis rate was 12.78 ± 1.64 (P=0.00087), 12.22 ± 1.73 (P=0.00080), 13.66 ± 4.07 (P=0.00274) and 11.18 ± 5.78 (P=0.00358) respectively. When compared with the apoptosis model group, these values were significantly reduced (P<0.01). However, there was no obvious influence on moderate and late cell apoptosis or on cell necrosis (P>0.05).

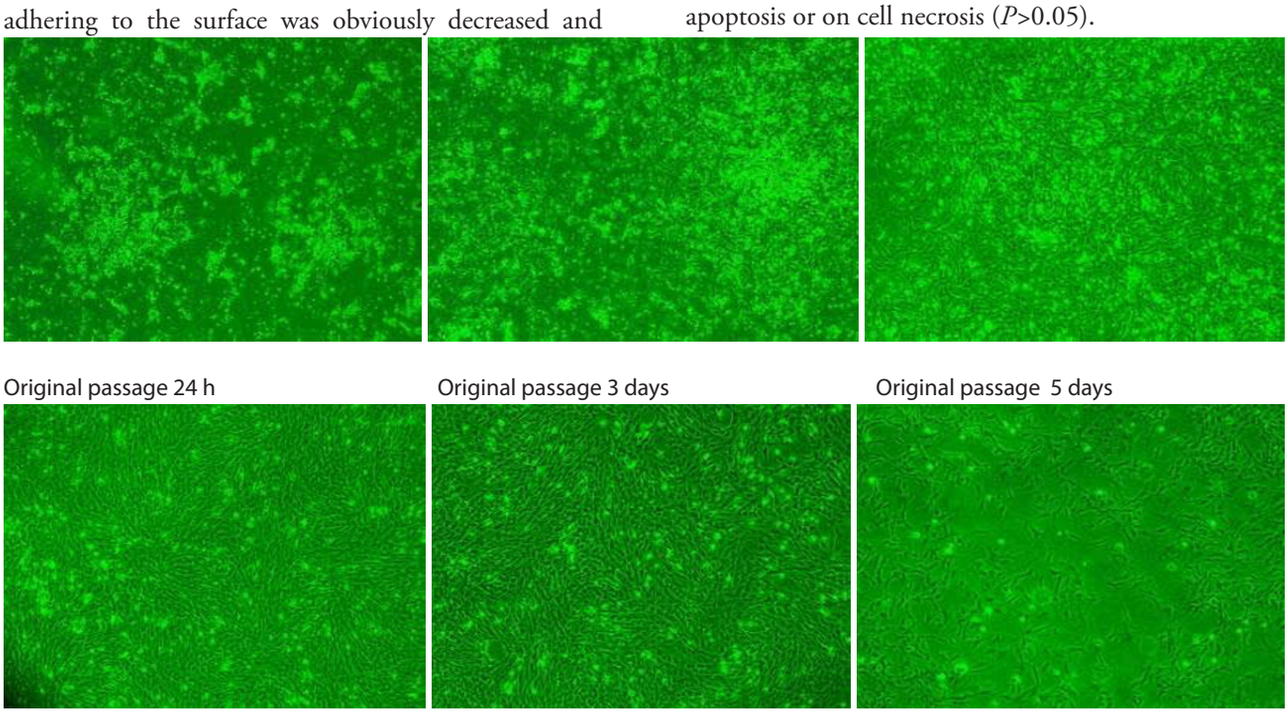


Figure 1 Cultured rat BMSCs (50×) observed under an inverted microscope.

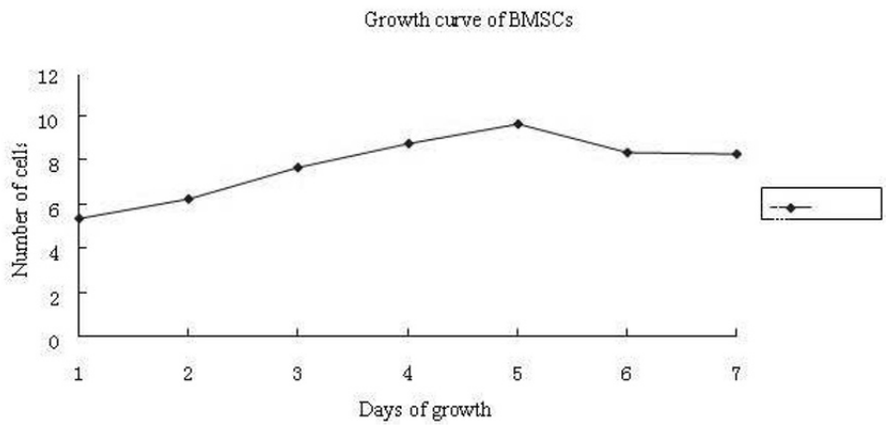
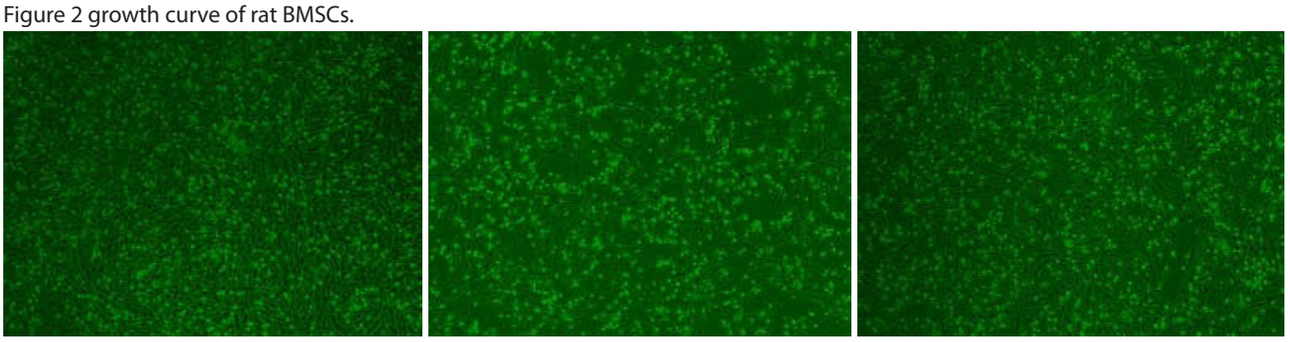
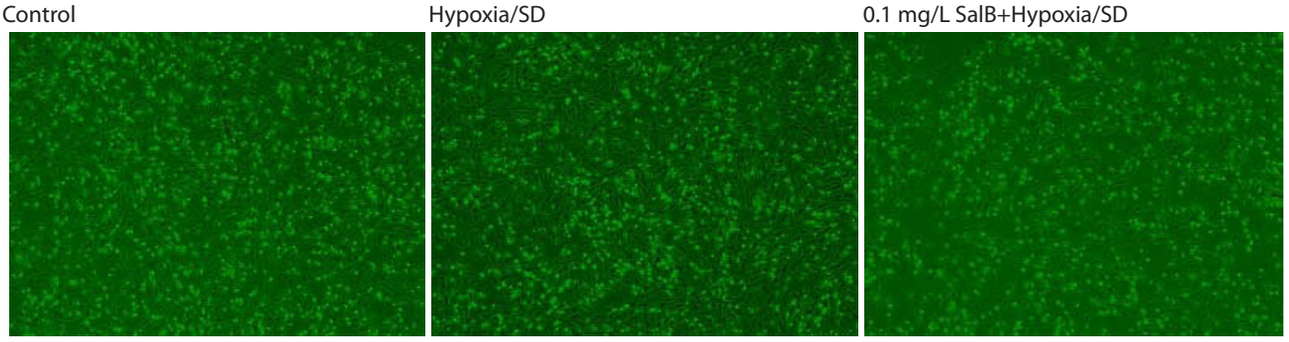


Figure 2 growth curve of rat BMSCs.

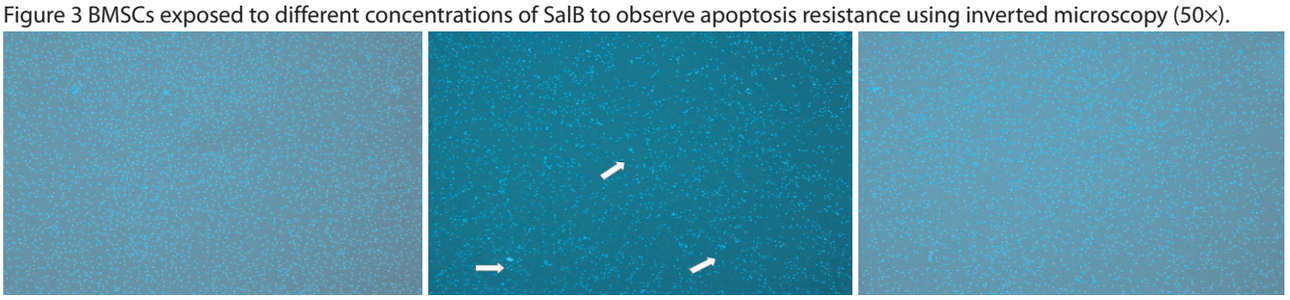


Control | Hypoxia/SD | 0.1 mg/L SalB+Hypoxia/SD

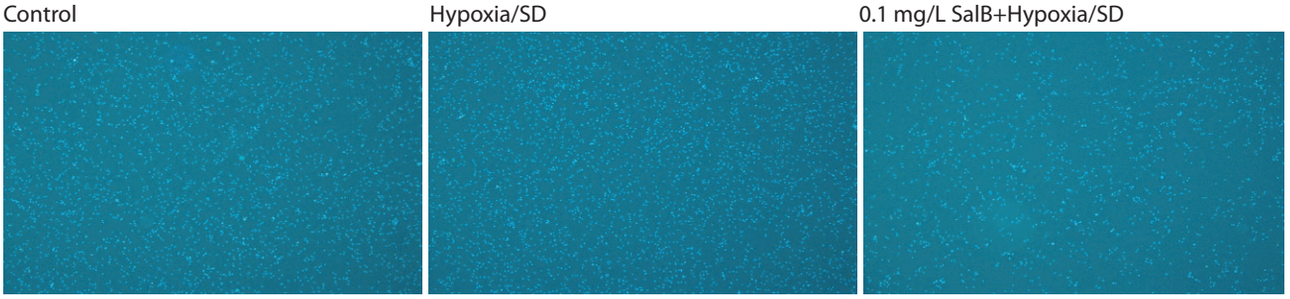


1 mg/L SalB+Hypoxia/SD | 10 mg/L SalB+Hypoxia/SD | 100 mg/L SalB+Hypoxia/SD

Figure 3 BMSCs exposed to different concentrations of SalB to observe apoptosis resistance using inverted microscopy (50×).



Control | Hypoxia/SD | 0.1 mg/L SalB+Hypoxia/SD



1 mg/L SalB+Hypoxia/SD | 10 mg/L SalB+Hypoxia/SD | 100 mg/L SalB+Hypoxia/SD

Figure 4 BMSCs exposed to SalB observed for apoptosis resistance using Hoechst 33342 and fluorescence microscopy (50×).

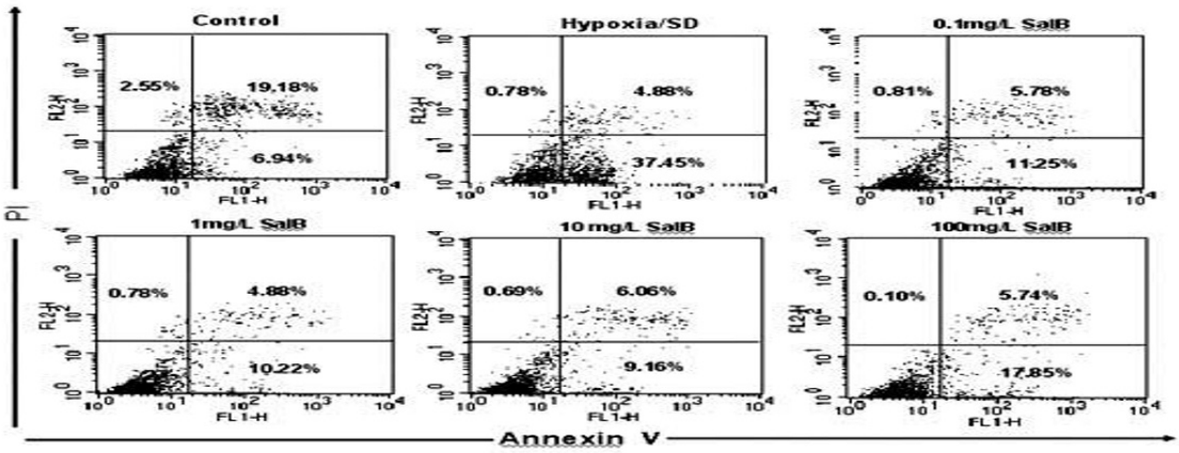


Figure 5 AnnexinV/PI double dyed flow cytometry used to detect SalB apoptosis resistance in BMSCs.

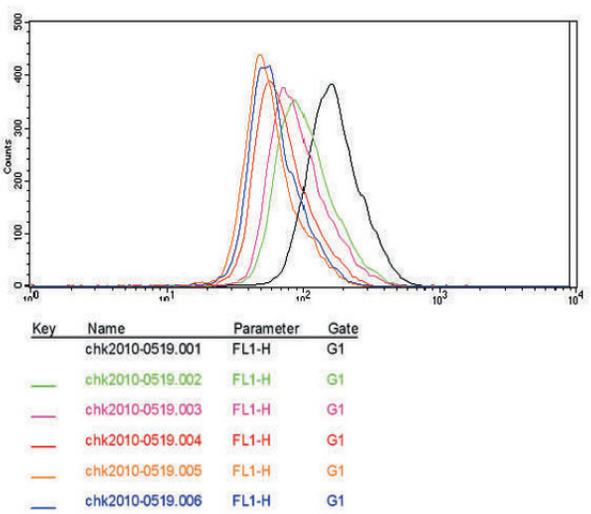


Figure 6 Flow cytometry to detect the influence of SalB on MMP in apoptosis induced BMSCs.

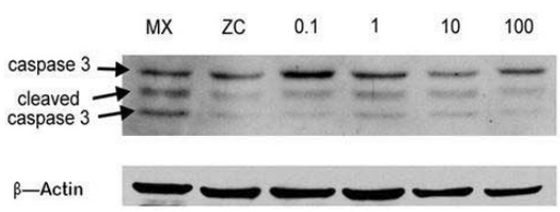


Figure 7 SalB inhibits activation of caspase-3 induced by hypoxia/SD.

Determining the mechanism of SalB apoptosis resistance in BMSCs induced by hypoxia/SD

Influence of SalB on MMP of apoptotic cells: The strength of fluorescence after the combination of Rhodamine 123 with the mitochondrial matrix, indicates the increase or decrease in negative MMP. As shown in Figure 6, compared with the apoptosis model group, SalB-treated groups had increased MMP, indicating that the ability of SalB to inhibit apoptosis induced by hypoxia/SD is guided by mitochondria.

SalB inhibits the activation of caspase-3 induced by hypoxia/SD: Western blot analysis was used to detect the activation of caspase-3. As shown in Figure 7, there was no activation of caspase-3 in control cells. In the apoptosis model group, caspase-3 was expressed in large amounts, indicative of apoptosis. Compared with the apoptosis model group, groups treated with 0.1, 1, 10 and 100 mg/L of SalB had reduced levels of caspase-3, indicating that SalB inhibits apoptosis to some extent.

DISCUSSION

Treatment of ischemic heart disease, especially myocardial infarction and other severe diseases, with stem cell grafting has drawn much attention from clinical experts, becoming a prominent research area in recent years. However, low survival rates of grafted BMSCs has hindered its use. Enhancing the survival rate of grafted BMSCs is a major clinical need that must be addressed. It has been reported that the lipid-reducing drugs, Lovastatin and glucocorticoid hormone (hydrocortisone), can effectively inhibit early apoptosis of BMSCs induced by hypoxia/SD in vitro[4,5].

The traditional Chinese medicine drug, Danshen (Radix Salviae Miltiorrhizae), is widely used to treat diseases of the cardiovascular system and has achieved good curative effects in ischemic heart disease. It acts to expand coronary arteries, increase the blood flow through coronary arteries, improve micro-circulation, reduce the amount of oxygen consumption in cardiac muscles and prevent and treat myocardial ischemia and myocardial infarction [6-8]. It has been verified that SalB, a main ingredient of Danshen, can promote stem cell division and inhibit cell apoptosis in many cell types [9,10]. At present however, there are no reports as to whether SalB can inhibit apoptosis in BMSCs, which could be significant in enhancing the survival rate of grafted BMSCs.

Throughout this research, hypoxia/SD was used to imitate the micro-environment of ischemic cardiac muscle. This was achieved in vitro using a BMSCs apoptosis model of BMSCs. A 6 h time point was selected as

the time of treatment with hypoxia/SD, where the highest early BMSC apoptosis rate is observed. SalB was investigated as an intervention drug to determine its effect on apoptosis inhibition in BMSCs.

Cell apoptosis is characterized by cell shrinkage and agglutination of karyon chromatin into plaques around the nuclear membrane, resulting in a fragmented appearance. Morphological results obtained by microscopy and Hochest 33342 fluorescent dye, show that treatment with 0.1, 1 and 10 mg/L SalB results in reduced apoptosis in BMSCs and inhibited nuclear shrinkage and fragmentation.

Statistical analysis of flow cytometry data indicate that BMSC early apoptosis rates in the groups treated with 0.1, 1, 10 and 100 mg/L SalB are significantly lower than in the apoptosis model group (P>0.01). However, there is no obvious influence on BMSC moderate or late apoptosis rates or on necrotic cells (P>0.05). When using 100 mg/L of SalB, an anti-apoptosis effect was not observed. In light of these data, we draw the following conclusion: SalB at concentrations of 0.1-10 mg/L can inhibit early apoptosis in BMSCs induced by hypoxia/SD, but has no effect on moderate or late apoptosis.

The mitochondrion is a "factory" of energy metabolism throughout the whole body and plays an important role in cell apoptosis. Multiple stimuli contribute to cell apoptosis. The reduction in MMP is considered to be the earliest event during the process of cell apoptosis, before characteristics of nuclear apoptosis appear (concentration of chromatin and break of DNA). Once the mitochondrial membrane potential ($\Delta\Psi$m) has collapsed, cell apoptosis is irreversible.

Cytochrome C exists mainly in the gap of the mitochondrial membranes. Various apoptosis-stimulating factors can induce its release into the cytoplasm to initiate the process of apoptosis induced by caspase[11]. Previous researchers have reported that protection of mitochondrial function can obstruct cell apoptosis[12]. Research into the genes that regulate and control cell apoptosis has been widely focused on Bcl-2 and Caspase-3[13]. It is confirmed that Bcl-2 exists on the mitochondrial membrane, nuclear membrane and endoplasmic reticular membrane. Caspase is considered the most important proteinase in the process of cell apoptosis, hence acquiring the name, death proteinase. Among the 14 family members discovered to date, caspase-3 is the most important proteinase. The activation of caspase-3 is a crucial downstream event within apoptotic cascade. Activated caspase-3 functioned as the important executioner of apoptosis.

The experimental result shows that SalB may inhibit the reduction in MMP caused by hypoxia/SD and reduce the activation of caspase-3 to inhibit BMSC apoptosis, thus further verifying the important role of protecting mitochondrial function for inhibiting apoptosis.

In summary, this is the first report that SalB, a single ingredient of the traditional Chinese medicine drug, Danshen, promotes apoptosis-resistance in BMSCs induced by hypoxia/SD. This provides a novel approach for enhancing the survival rate of grafted stem cells and improving the curative effect of the stem cell graft in ischemic heart disease, with great potential for clinical use.

In the treatment of myocardial infarction and other ischemic heart diseases with stem cell grafts, SalB could be used to inhibit apoptosis of stem cells, to enhance the survival rate of grafted stem cells and improve the curative effect within clinical practice. Based on our results, the following approach is suggested: 1) Prior to stem cell grafting, pre-treat cells with SalB to enhance the survival rate of the grafted cells. 2) Prior to stem cell grafting, patients should begin to take or be injected with a drug containing SalB as its main ingredient. This will ensure levels of SalB in the body reach effective drug concentration for inhibiting apoptosis of stem cells, clinically enhancing the curative effect of the stem cell graft.

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